Local drug and gene delivery through microbubbles and ultrasound: a safe and efficient alternative for viral vectors?

L.J.M. Juffermans, P.A. Dijkmans, R.J.P. Musters, A. van Wamel, A. Bouakaz, F.J. ten Cate, L. Deelman, C.A. Visser, N. de Jong, O. Kamp

Although gene therapy has great potential as a treatment for diseases, clinical trials are slowed down by the development of a safe and efficient gene delivery system. In this review, we will give an overview of the viral and nonviral vehicles used for drug and gene delivery, and the different nonviral delivery techniques, thereby focusing on delivery through ultrasound contrast agents.
The development of ultrasound contrast agents containing encapsulated microbubbles has increased the possibilities not only for diagnostic imaging, but for therapy as well. Microbubbles have been shown to be able to carry drugs and genes, and destruction of the bubbles by ultrasound will result in local release of their contents. Furthermore, ligands can be attached so that they can be targeted to a specific target tissue. The recent advances of microbubbles as vehicles for delivery of drugs and genes will be highlighted. (*Neth Heart J* 2004;12:398-403.)

Key words: drug delivery, gene therapy, microbubbles, nonviral, ultrasound, viral

L.J.M. Juffermans
P.A. Dijkmans
R.J.P. Musters
C.A. Visser
O. Kamp
Institute for Cardiovascular Research, VU University Medical Centre, Amsterdam
A. van Wamel
A. Bouakaz
F.J. ten Cate
N. de Jong
Erasmus Medical Centre, Thorax Centre, Rotterdam
L. Deelman
Groningen University Hospital, Groningen

Correspondence to: L.J.M. Juffermans
Laboratory for Physiology, VU University Medical Centre,
Van der Boechorststraat 7, 1081 BT Amsterdam
E-mail: ljm.juffermans@vumc.nl

As a consequence of the identification of thousands of genetic factors, gene therapy will have great potential as a treatment for several (cardiovascular) diseases in the near future. However, clinical trials are slowed down by the development of safe and efficient systems for local gene delivery. At present two categories of delivery vehicles are available: viral and nonviral. Up till now, the use of viruses has been the most efficient gene transfer method because of the specific viral machinery that has evolved to introduce viral DNA into their host cells.[1] However, there are many problems regarding the safety of viral vectors. The ideal delivery system should be safe, i.e. elicit no immune response, and efficient, i.e. be able to transfect the majority of cells. There should be no breakdown of DNA before it reaches the target, the system should be able to bypass the endosomal degradation route and there should be sustained transgene expression. Besides this, the amount of DNA or drugs needed to be effective should be minimal because of possible side effects and it would be convenient if it could be commercially produced on a large scale.[2-4]

Viral vehicles

Enveloped viruses accomplish the delivery of their genome into the host cell by fusing their envelopes with the host cell membrane, mediated by proteins incorporated in the viral envelope. Conversely, viruses that enter via endocytosis have specific proteins to destabilise the endosomal membrane to enable them to escape the lysosomal degradation route.[5,6] As viral DNA often contains several nuclear localisation sequences it will be imported into the nucleus, followed by integration into the host's genome via integration sites on the viral DNA.[7] Particularly retroviruses, lentiviruses and adenoviruses or adeno-associated viruses have been extensively used as vehicles.[2]

Retroviral vectors

Retroviral vectors were the first to be designed, and the development of self-inactivating vectors in which the viral regulatory elements have been deleted was a breakthrough in generating a safe and transcriptionally adjustable vector. The main limitation of retroviral vectors is their inability to infect nondividing cells, meaning that

tissues such as brain, eye and lung are not amenable for direct in vivo gene delivery. Furthermore, on transplantation of transduced cells in the host, transcription of the transgene is often 'switched off' and the transgene is no longer expressed.[2]

Lentiviral vectors

Derived from the human immunodeficiency virus, lentiviral vectors belong to the retrovirus family but have acquired the property of infecting nondividing cells, which is unusual for retroviruses. Although the first-generation lentiviral vectors were promising, the possibility remained that they could recombine and generate the dangerous immunodefiency virus. To minimise this chance, as many viral accessory genes as possible were deleted, while maintaining the capacity of infecting nondividing cells. This technique is also applied to the adenoviral vectors, for example, to create a safe 'gutless' virus. However, there are still no reports of clinical trials using lentiviral vectors as they have the disadvantage of nonspecific integration in the host's genome, which could theoretically lead to activation of pro-oncogenes.[2]

Adeno-associated viral vectors

The most promising viral vectors are adeno-associated. The adeno-associated virus is nonpathogenic and is a member of the dependoviruses, meaning that it needs extra genes to replicate. These genes are provided by the adenovirus or herpesvirus. The broad host range, relatively low level of immune response, longevity of gene expression, and ability to integrate site-specifically have enabled a number of clinical trials to be initiated. The main disadvantage of using adeno-associated viral vectors is that the gene that enables the site-specific integration is cytostatic or even cytotoxic to the host cells. Another drawback of using this vector for gene therapy is that it can only code for a transgene of maximal 4.5 kb.[2,8]

Adenoviruses

Adenoviruses belong to a family of DNA tumour viruses. Advantages of adenoviral vectors are that they retain the ability to transduce dividing and nondividing cells efficiently and the relative easiness to generate high-titre commercial-grade recombinant vectors. The problem with adenoviral vectors is that the expression of the transgene in adult animals only lasts for a short period of time (5-20 days postinfection), which is now generally recognised as a consequence of the immune response. The immune response of the host is the biggest challenge facing all viral vectors. Even inactivated adenoviral vectors can elicit a strong immune response: a few years ago an 18-year-old patient died as a direct consequence of the severe immune response to the adenoviral vector used in a phase I gene therapy clinical trial.[2,8]

Nonviral delivery techniques

As viral vectors bring along many problems associated with immunity, a nonviral yet efficient gene delivery method is desirable. The following nonviral gene delivery systems are now available.

Microinjection

DNA transfer is performed by direct injection of naked DNA into a cell nucleus. This is a very effective method but as only one cell at the time can be transfected, its use is limited.[2,4]

Gene gun

Nonviral gene delivery can also be performed with the so-called 'gene gun', where DNA-coated gold particles are accelerated to a high velocity and shot under helium pressure into the skin.[4] This method was developed by Sanford and colleagues in 1987 to deliver genes to plant cells. The gene gun is a convenient device that provides rapid and direct gene transfer into a range of targets in vitro as well as in vivo.[9] However, this procedure is not yet widely used because the DNA entry pathway is difficult to control.[4]

Electroporation

Another relatively effective method is electroporation. Electrical pulses are used to transiently permeabilise the cell membrane, thus permitting cellular uptake of macromolecules. This process was first used to deliver DNA in vitro to mammalian cells as early as in 1982. It is one of the most efficient methods to transfect skeletal muscle cells, but its use is limited because of the high mortality of cells after high-voltage exposure and the difficulty to apply in vivo. Also, the efficacy in humans has yet to be tested.[10]

Magnetofection

A novel delivery technique under development uses the magnetic force acting on gene vectors that are associated with magnetic particles. Magnetofection does not necessarily improve overall performance of any given standard gene transfer method in vitro, its major potential lies in the very rapid and efficient transfection at low vector doses and the possibility to remotely control vector targeting in vivo. Although this technique is still in its infancy, it has great potential.[11,12]

Ultrasound contrast agents

Another new technique is the use of ultrasound contrast agents, which is characterised by the local delivery of genes and/or drugs through microbubbles and ultrasound to specific target tissues, including the heart.[13-16]

Nonviral vehicles

During the last decade much attention has been focused on the use of various (phospho)lipid complexes and formulations as vehicles for drug and gene delivery.[11-20]

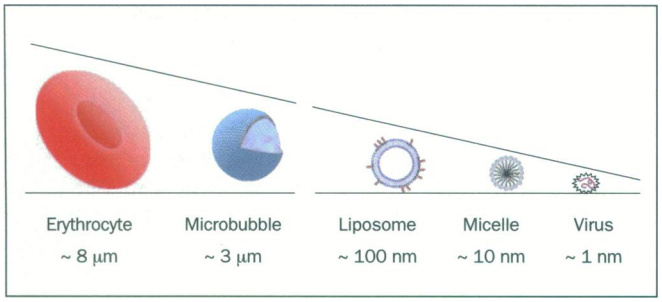

Figure 1. Alignment of the different particles according to their size, starting with an erythrocyte for size comparison.

Micelles

Micelles are mostly used as carriers to deliver drugs in anticancer therapy; they are phospholipid particles and about 10 to 100 nm in diameter, approximately ten times bigger than a virus, as can be seen in figure 1.[17]

Liposomes

Liposomes have a phospholipid bilayer and are on average only 100 nm in diameter. Liposomes were a promising tool in gene and drug delivery, but although in vitro studies do show good results, in vivo studies are disappointing.[18,19]

Microbubbles

Gramiak and Shah were the first to notice an elevated signal after an intracardiac infection of saline, although it was later that they attributed this to the reflection of ultrasound by minibubbles in the saline.[20] Since then, microbubbles have evolved rapidly, not only in diagnostic imaging but as possible therapeutic agents as well.[14] Microbubbles are on average about 2 to 5 µm in diameter, which is relatively large compared with other carriers used in gene and drug delivery, such as the mentioned particles for magnetofection which are only 10 to 20 nm.[11,12] The very first microbubbles manufactured were room air microspheres. Albunex was the first contrast agent to be approved for clinical use in the United States and Japan; it consists of air stabilised with a thin shell of human albumin.[21] However, air bubbles disappear in a few seconds after intravenous administration as the solubility of air in blood is high and the bubbles are filtered by the lungs.[22-24] Improved stability and survival was reached by stabilising the microbubbles with a high molecular weight gas, such as sulphur hexafluoride, which decreases solubility and thus improves survival and stability under high pressure. Various ultrasound contrast agents are now commercially available with different gases and different types of shells; a few well-known microbubbles and their characteristics are shown in table 1, and figure 2 shows fluorescently labelled microbubbles.

Microbubble properties: an ideal vehicle

An interesting feature of microbubbles is the specific acoustic properties they show in the presence of ultrasound, due to the encapsulated gas. This provides various possible methods of increasing cell permeability by these ultrasound microbubbles.[25,26]

Firstly, an important mechanism in permeabilising cell membrane is cavitation: the interaction between the ultrasound and the surrounding fluid.[26,27] In body tissue or blood, cavitation sets fluid in motion and creates small shock waves that give rise to microstreaming and shear stress along the endothelial cells.[26-28] It is hypothesised that microbubbles acting as cavitation nuclei lower the threshold for cavitation, potentiating the formation of transient holes in the plasma membrane and possibly the nuclear membrane, which would facilitate macromolecule uptake.[10,27]

Ultrasound applied at low or intermediate acoustic power (MI <0.5) results in linear and nonlinear oscillations of microbubbles, respectively, inducing stable cavitation.[27] When microbubbles are exposed to ultrasound with high acoustic power, i.e. MI >1.0, it will lead to nonlinear expansion and compression of the microbubbles with high oscillation amplitude, eventually leading to bubble destruction.[29] When

Table 1. Classification of nonviral vehicles and ultrasound microbubbles.

Nonviral vehicles	Inside	Shell	Mean size
Nanoparticles		Latex	10–20 nm
Micelles	Surrounding solvent	Phospholipid particle	10–100 nm
Liposomes	Surrounding solvent	Phospholipid bilayer	~100 nm
Microbubbles			
First-generation Echovist[a]	Air	Galactose matrix	~2 µm
Second-generation Albunex[b]	Air	Human albumin	1.5–6 µm
Third-generation Optison[a]	Perfluoropropane	Human albumin	2–4.5 µm
Sonovue[a]	Sulphur hexafluoride	Phospholipid monolayer	2–5 µm
PESDA	Perfluorobutane	Sonicated albumin	2–5 µm
Quantison	Air	Dried albumin	2–5 µm
Definity	Perfluoropropane	Phospholipid monolayer	~2.5 µm

[a]Licensed for clinical use. [b]No longer commercially available.

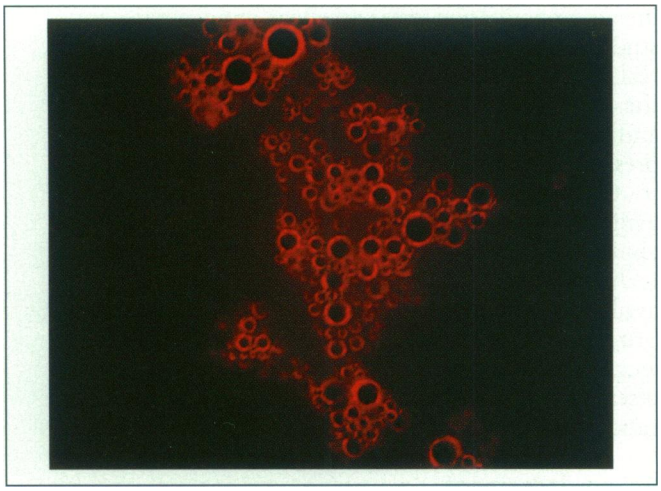

Figure 2. Fluorescently labelled SonoVue microbubbles.

microbubbles are destroyed they may act as 'fragmentation bombs' (figure 3), shooting the genes into the cells. Price and colleagues showed that microbubble destruction is able to create microvessel ruptures large enough to permit the extravasation of microbubbles, yet cell and tissue damage are minimal and limited to the rupture site itself. This study shows that microbubbles in the presence of ultrasound can be used to deliver genes across the endothelial lining of the blood vessel to the underlying tissue.[13] Local release of the bubble's cargo by destruction can be well used for the local delivery of a high concentration of drugs to target tissue. All forms of drug therapy in which drugs are administered systemically require plasma concentrations within the therapeutic range. Although many diseases such as cancer, inflammatory diseases or thromboembolic processes may require higher concentrations of certain drugs, plasma concentrations are limited by the occurrence of potentially dangerous side effects. After systemic administration, microbubbles loaded with drugs can rupture under influence of localised ultrasound and drug release will result in higher local concentrations in comparison with systemic administration.[30]

Secondly, changes in the plasma membrane fluidity may facilitate microbubble and/or DNA uptake. When diagnostic cardiac ultrasound is applied oxygen radicals are produced in the endothelial cells.[31] This could lead to an increased permeability of the plasma membrane; however, a dramatic increase in oxygen radicals may also have toxic effects. Thirdly, ultrasound in the presence of microbubbles causes a local, transient rise in temperature.[32] This could also contribute to an increased fluidity and permeability of the cell membrane.

A fourth cell entry mechanism is the fusion of the phospholipid-shelled microbubble or exchange of bubble fragments with the plasma membrane, delivering the bubble's cargo direct into the cytoplasm of the cell. Or finally, microbubbles may be actively taken up via phagocytosis or endocytosis. This is most likely to happen when the microbubbles are targeted; when a microbubble binds to, for example, the transferrin receptor, endocytosis is triggered. As they often have a net positive charge, nontargeted microbubbles may also bind to the negatively charged cell surface molecules and trigger endocytosis.[33]

Targeting the microbubbles

As mentioned briefly, microbubbles are ideal delivery vehicles as they can be loaded with genes or drugs, but besides that they can be targeted to specific tissue by the incorporation of ligands into the shell. There are several ways in which drug, genes or ligands attach to a microbubble (figure 4). Especially negatively charged DNA can rather easily be attached to a positively charged bubble shell (figure 4A). Another way to load a bubble with drugs is to incorporate a lipophilic drug in the lipid membrane (figure 4B), or to enclose the drug within the microbubble itself (figure 4C). Furthermore, drugs can be bound by ligands that are embedded in the membrane (figure 4D). Whether a bubble can be loaded with a certain drug depends on important factors such as molecular weight, lipophilicity and charge.[34]

Recent experiments show that it is possible to create targeted microbubbles by incorporating monoclonal antibodies into the membrane.[35-38] Villanueva and colleagues demonstrated that microbubbles with intercellular adhesion molecule-I (ICAM-I) antibodies bind to endothelial cells expressing ICAM-I. As the expression of ICAM-I by endothelial cells is associated with early arteriosclerosis, this has major consequences for diagnosis of preclinical atherosclerosis.[35] Schumann and colleagues demonstrated that vascular clots, known to be associated with cardiovascular diseases such as stroke and myocardial infarction, could be visualised by microbubbles targeted to the GP IIb/IIIa receptors, which play a key role in the formation of vascular clots. Clot lysis is known to be improved by ultrasound, which creates the possibility of enhanced clot lysis by ultrasound in combination with targeted

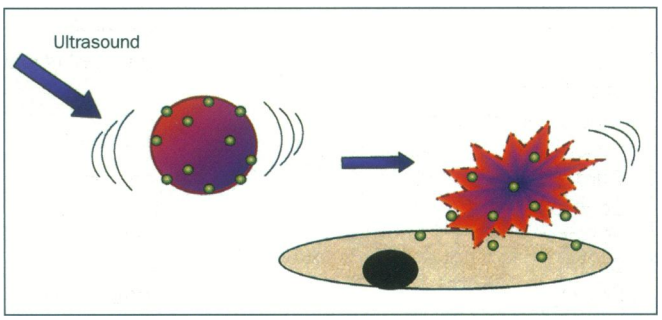

Figure 3. Microbubble collapse under influence of ultrasound, thereby perforating the cell membrane.

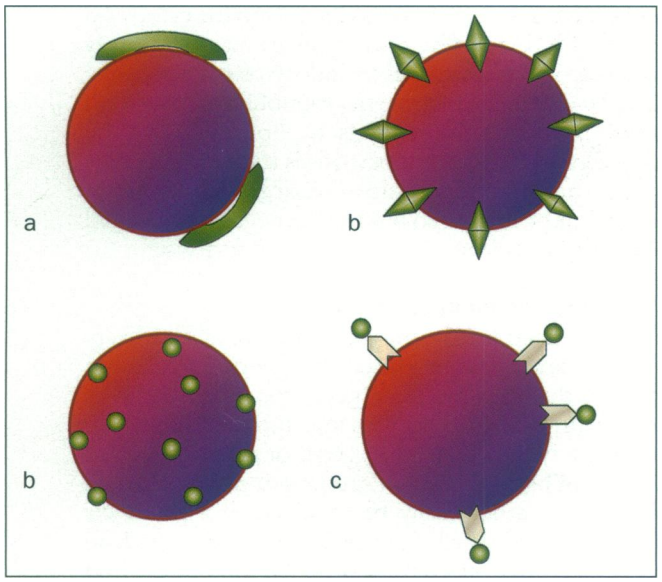

Figure 4. Different ways to bind drugs, genes or ligands to a microbubble.

microbubbles loaded with thrombolytics.[37,39] Although many studies implicate that microbubbles can be used as vehicles for targeted drug and gene delivery, it is important to know how exactly cell entry occurs under different circumstances and what the bioeffects are that microbubbles and ultrasound can cause, before this technique can be used as a safe and efficient tool to deliver genes or drugs in humans. Adverse bioeffects, such as a rise in blood temperature or haemolysis when using ultrasound and microbubbles, have been causes of concern for several investigators. Microbubbles exposed to ultrasound can cause mechanical stress and damage to cells and consequently even permanent cell injury.[40] The extent of the bioeffects that therapeutic ultrasound microbubbles can result in depends on ultrasound parameters such as frequency and amplitude, which are lower for diagnostic ultrasound compared with therapeutic ultrasound.[26,32,41,42] Implications for side effects in humans and their clinical importance need further investigation.

Perspectives

Different approaches to nonviral gene and drug delivery are being explored, and much has been learned from viruses that have evolved into extremely efficient infection mechanisms. Recent in vivo studies have shown that enhanced expression of genes delivered by microbubbles in combination with ultrasound is feasible. The most interesting is the precise interaction of microbubbles with living cells. Although several options such as transient cell membrane holes, phagocytosis, endocytosis and fusion of microbubble shell components with the cell membrane have been proposed, the exact mechanism(s) remain(s) to be unravelled. Recent advances in live-cell imaging techniques, for example multidimensional digital imaging microscopy, offer excellent opportunities to study in vitro this process at the (sub)cellular level in real-time, thereby creating the possibility of visualising the interaction of fluorescent-labelled microbubbles and myocardial or endothelial cells under ultrasound pressure.

Over the past few years, contrast agents have rapidly evolved from a diagnostic to a possible therapeutic application. In the coming years, this promising technique needs further development to make it available for safe and efficient local gene therapy and drug delivery as this technique creates various challenging therapeutic options, not only in cardiovascular disease but also in the treatment of different types of cancer. ■

Acknowledgement

This study is supported and performed by the Interuniversity Cardiology Institute of the Netherlands (ICIN/KNAW), project 49.

References

1 Verma IM, Somia N. Gene therapy--promises, problems and prospects. *Nature* 1997;**389**:239-42.
2 Somia N, Verma IM. Gene therapy: trials and tribulations. *Nat Rev Genet* 2000;**1**:91-9.
3 Luo D, Saltzman WM. Synthetic DNA delivery systems. *Nat Biotechnol* 2000;**18**:33-7.
4 Schmidt-Wolf GD, Schmidt-Wolf IG. Non-viral and hybrid vectors in human gene therapy: an update. *Trends Mol Med* 2003;**9**:67-72.
5 Fredericksen BL, Wei BL, Yao J, Luo T, Garcia JV. Inhibition of endosomal/lysosomal degradation increases the infectivity of Human Immuno-deficiency Virus. *J Virol* 2002;**76**:11440-6.
6 Murthy N, Campbell J, Fausto N, Hoffman AS, Stayton PS. Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. *J Control Release* 2003;**89**:365-74.
7 Darnell J, ed. Molecular cell biology. Scientific American Books, Inc., 1995.
8 Zhao N, Liu DP, Liang CC. Hot topics in adeno-associated virus as a gene transfer vector. *Mol Biotechnol* 2001;**19**:229-37.
9 Conn A, Durrant L, Spendlove I. Delivery of pCMV-S DNA using the Helios gene gun system is superior to intramuscular injection in Balb/c mice. www.bio-rad.com.
10 Lu QL, Bou-Gharios G, Partridge TA. Non-viral gene delivery in skeletal muscle: a protein factory. *Gene Ther* 2003;**10**:131-42.
11 Plank C, Schillinger U, Scherer F, Bergemann C, Remy JS, Krotz F, et al. The magnetofection method: using magnetic force to enhance gene delivery. *Biol Chem* 2003;**384**:737-47.
12 Pulfer SK, Ciccotto, SL, Gallo JM. Distribution of small magnetic particles in brain tumor-bearing rats. *J Neurooncol* 2003;**41**:99-105.
13 Price RJ, Skyba DM, Kaul S, Skalak TC. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation* 1998;**98**:1264-7.
14 Sieswerda GT, Kamp O, Visser CA. The use of contrast agents in echocardiography - part II: Clinical indications and experimental applications. *Cardiologie* 1998;**5**:648-57.
15 Sieswerda GT. Clinical studies in contrast-echocardiography: from left ventricular opacification to evaluation of myocardial perfusion [thesis]. Free University of Amsterdam, 2003.
16 Bekeredjian R, Chen S, Frenkel PA, Grayburn P, Shohet RV. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. *Circulation* 2003;**108**:1022-6.
17 Marin A, Sun H, Husseini G, Pitt W, Christensen D, Rapoport N. Drug delivery in pluronic micelles: effect of high-frequency

ultrasound on drug release from micelles and intracellular uptake. *J Control Release* 2002;**84**:39-47.
18 Li S, Huang L. Nonviral gene therapy: promises and challenges. *Gene Ther* 2000;7:31-4.
19 Maurer N, Fenske DB, Cullis PR. Developments in liposomal drug delivery systems. *Expert Opin Biol Ther* 2001;**1**:923-47.
20 Gramiak R, Shah PM. Echocardiography of the aortic root. *Invest Radiol* 1968;**3**:356-66.
21 Barnhart J, Levene H, Villapando E, Maniquis J, Fernandez J, Rice S, et al. Characteristics of Albunex: air-filled albumin microspheres for echocardiography contrast enhancement. *Invest Radiol* 1990;**25**:162-4.
22 Bouakaz A, de Jong N, Cachard C, Jouini K. On the effect of lung filtering and cardiac pressure on the standard properties of ultrasound contrast agent. *Ultrasonics* 1998;**36**:703-8.
23 Mayer S, Grayburn PA. Myocardial contrast agents: recent advances and future directions. *Prog Cardiovasc Dis* 2001;**44**:33-44.
24 Correas JM, Bridal L, Lesavre A, Mejean A, Claudon M, Helenon O. Ultrasound contrast agents: properties, principles of action, tolerance, and artefacts. *Eur Radiol* 2001;**11**:1316-28.
25 Porter TR, Xie F. Therapeutic ultrasound for gene delivery. *Echocardiography* 2001;**18**:349-53.
26 Van Wamel A, Bouakaz A, Houtgraaf J, ten Cate J, de Jong N. Effects of diagnostic ultrasound parameters on molecular uptake and cell viability in ISTU (Seattle, 2002).
27 Lawrie A, Brisken AF, Francis SE, Cumberland DC, Crossman DC, Newman CM. Microbubble-enhanced ultrasound for vascular gene delivery. *Gene Ther* 2000;7:2023-7.
28 Wu J. Theoretical study on shear stress generated by microstreaming surrounding contrast agents attached to living cells. *Ultrasound Med Biol* 2002;**28**:125-9.
29 Chen S, Shohet RV, Bekeredjian R, Frenkel P, Grayburn PA. Optimization of ultrasound parameters for cardiac gene delivery of adenoviral or plasmid deoxyribonucleic acid by ultrasound-targeted microbubble destruction. *J Am Coll Cardiol* 2003;**42**:301-8.
30 Hunter WL, Burt HM, Machan L. Local delivery of chemotherapy: a supplement to existing cancer treatments. A case for surgical pastes and coated stents. *Adv Drug Deliv Rev* 1997;**26**:199-207.
31 Basta G, Venneri L, Lazzerini G, Pasanisi E, Pianelli M, Vesentini N, et al. In vitro modulation of intracellular oxidative stress of endothelial cells by diagnostic cardiac ultrasound. *Cardiovasc Res* 2003;**58**:156-61.
32 Wu J. Temperature rise generated by ultrasound in the presence of contrast agent. *Ultrasound Med Biol* 1998;**24**:267-74.
33 Zuhorn IS, Hoekstra D. On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? *J Membr Biol* 2002;**189**:167-79.
34 Unger EC, Matsunaga TO, McCreery T, Schumann P, Sweitzer R, Quigley R. Therapeutic applications of microbubbles. *Eur J Radiol* 2002;**42**:160-8.
35 Villanueva FS, Jankowski RJ, Klibanov S, Pina ML, Alber SM, Watkins SC, et al. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells. *Circulation* 1998;**98**:1-5.
36 Lindner JR, Song J, Xu F, Klibanov AL, Singbartl K, Ley K, et al. Noninvasive ultrasound imaging of inflammation using microbubbles targeted to activated leukocytes. *Circulation* 2000;**102**:2745-50.
37 Schumann PA, Christiansen JP, Quigley RM, McCreery TP, Sweitzer RH, Unger EC, et al. Targeted-microbubble binding selectively to GPIIbIIIa receptors of platelet thrombi. *Invest Radiol* 2002;**37**:587-93.
38 Unger EC, Hersh E, Vannan M, Matsunaga TO, McCreery T. Local drug and gene delivery through microbubbles. *Prog Cardiovasc Dis* 2001;**44**:45-54.
39 Leong-Poi H, Christiansen J, Klibanov AL, Kaul S, Lindner JR. Noninvasive assessment of angiogenesis by ultrasound and microbubbles targeted to alpha(v)-integrins. *Circulation* 2003;**107**:455-60.
40 Van Wamel A, Bouakaz A, de Jong N. Duration of ultrasound bubbles enhanced cell membrane permeability. IEEE International Ultrasonics Symposium Hawaii. USA 2003.
41 Miller DL, Gies RA. Enhancement of ultrasonically induced hemolysis by perfluoro-carbon-based compared to air-based echo-contrast agents. *Ultrasound Med Biol* 1998;**24**:285-92.
42 Poliachik SL, Chandler WL, Mourad PD, Bailey MR, Bloch S, Cleveland RO, et al. Effect of high-intensity focused ultrasound on whole blood with and without microbubble contrast agent. *Ultrasound Med Biol* 1999;**25**:991-8.